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(71) Applicant *(for all designated States except US)*: SHEARWATER POLYMERS, INC. [US/US]; 2307 Spring Branch Road, Huntsville, AL 35801 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: HARRIS, J., Milton [US/US]; 3119 Highland Plaza, Huntsville, AL 35801 (US).

(74) Agents: PEDIGO, Paul, F. et al.; Bell Seltzer Intellectual Property Law Group, Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234 (US).

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(54) Title: DELIVERY OF POLY(ETHYLENE GLYCOL)–CONJUGATED MOLECULES FROM DEGRADABLE HYDROGELS

(57) Abstract

A degradable PEG hydrogel is described that, upon hydrolysis, releases conjugates of substantially non–peptidic polymers and biologically active molecules. For example, PEG and protein conjugates can be released in vivo from the hydrogels for therapeutic application.

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DELIVERY OF POLY (ETHYLENE GLYCOL) - CONJUGATED MOLECULES FROM DEGRADABLE HYDROGELS

Field of the Invention

This invention relates to crosslinked hydrogel networks that include the hydrophilic polymer poly(ethylene glycol).

Background of the Invention

Chemical attachment of the hydrophilic polymer poly(ethylene glycol)(PEG), also known as poly(ethylene oxide)(PEO), to molecules and surfaces is of great utility in biotechnology. In its most common form, PEG is a linear polymer terminated at each end with hydroxyl groups:

$$HO\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$$

This polymer can be represented in brief form as HO-PEG-OH where it is understood that the -PEG- symbol represents the following structural unit:

$$-CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2-$$

In typical form, n ranges from approximately 10 to approximately 2000.

PEG is commonly used as methoxy-PEG-OH, or mPEG in brief, in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group that is subject to ready chemical modification.

$$CH_3O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH \quad \text{mPEG}$$

PEG is also commonly used in branched forms that can be prepared by addition of ethylene oxide to

various polyols, such as glycerol, pentaerythritol and sorbitol. For example, the four-arm, branched PEG prepared from pentaerythritol is shown below;

$$C(CH_2\text{-}OH)_4 + n\ C_2H_4O \longrightarrow C[CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH]_4$$

The branched PEGs can be represented in general form as $R(\text{-}PEG\text{-}OH)_n$ in which R represents the central "core" molecule, such as glycerol or pentaerythritol, and n represents the number of arms.

PEG is a much used polymer having the properties of solubility in water and in many organic solvents, lack of toxicity, and lack of immunogenicity. One use of PEG is to covalently attach the polymer to insoluble molecules to make the resulting PEG-molecule "conjugate" soluble. For example, Greenwald, Pendri and Bolikal in J. Org. Chem., 60, 331-336 (1995) have shown that the water-insoluble drug taxol, when coupled to PEG, becomes water soluble.

Davis et al. U.S. Patent No. 4,179,337 describes proteins coupled to PEG and having enhanced blood circulation lifetime because of reduced rate of kidney clearance and reduced immunogenicity. The lack of toxicity of the polymer and its rapid clearance from the body are advantageous features for pharmaceutical applications. These applications and many leading references are described in the book by Harris (J. M. Harris, Ed., "Biomedical and Biotechnical Applications of Polyethylene Glycol Chemistry," Plenum, New York, 1992).

To couple PEG to a molecule such as a protein it is necessary to use an "activated derivative" of the PEG having a functional group at the terminus suitable for reacting with some group on the surface or on the protein (such as an amino group). Among the many useful activated derivatives of PEG is the succinimidyl "active ester" of carboxymethylated PEG as disclosed by

K. Iwasaki and Y. Iwashita in U.S. Patent No. 4,670,417. This chemistry is illustrated with the active ester reacting with amino groups of a protein (the succinimidyl group is represented as NHS and the protein is represented as PRO-NH_2):

$$PEG\text{-}O\text{-}CH_2\text{-}CO_2\text{-}NHS + PRO\text{-}NH_2 \longrightarrow PEG\text{-}O\text{-}CH_2\text{-}CO_2\text{-}NH\text{-}PRO$$

Succinimidyl "active esters", such as PEG-O-CH_2-CO_2-NHS, are commonly used forms of activated carboxylic acids, and they are prepared by reacting carboxylic acids with N-hydroxylsuccinimide.

Problems have arisen in the art. Some of the functional groups that have been used to activate PEG can result in toxic or otherwise undesirable residues when used for in vivo drug delivery. Some of the linkages that have been devised to attach functional groups to PEG can result in an undesirable immune response. Some of the functional groups do not have sufficient or otherwise appropriate selectivity for reacting with particular groups on proteins and can tend to deactivate the proteins.

PEG hydrogels, which are water-swollen gels, have been used for wound covering and drug delivery. PEG hydrogels are prepared by incorporating the soluble, hydrophilic polymer into a chemically crosslinked network or matrix so that addition of water produces an insoluble, swollen gel. Substances useful as drugs typically are not covalently attached to the PEG hydrogel for in vivo delivery. Instead, the substances are trapped within the crosslinked matrix and pass through the interstices in the matrix. The insoluble matrix can remain in the body indefinitely and control of the release of the drug can be somewhat imprecise.

One approach to preparation of these hydrogels is described in Embrey and Graham's U.S.

Patent No. 4,894,238, in which the ends of the linear polymer are connected by various strong, nondegradable chemical linkages. For example, linear PEG can be incorporated into a crosslinked network by reacting with a triol and a diisocyanate to form hydrolytically-stable ("nondegradable") urethane linkages.

A related approach for preparation of nondegradable PEG hydrogels has been demonstrated by Gayet and Fortier in J. Controlled Release, 38, 177-184 (1996) in which linear PEG was activated as the p-nitrophenylcarbonate and crosslinked by reaction with a protein, bovine serum albumin. The linkages formed are hydrolytically-stable urethane groups.

N.S. Chu U.S. Patent No. 3,963,805 describes nondegradable PEG networks have been prepared by random entanglement of PEG chains with other polymers formed by use of free radical initiators mixed with multifunctional monomers. P.A. King U.S. Patent No. 3,149,006 describes the preparation of nondegradable PEG hydrogels by radiation-induced crosslinking of high molecular weight PEG.

Nagaoka et al. U.S. Patent No. 4,424,311 describes PEG hydrogels prepared by copolymerization of PEG methacrylate with other comonomers such as methyl methacrylate. This vinyl polymerization will produce a polyethylene backbone with PEG attached. The methyl methacrylate comonomer is added to give the gel additional physical strength.

Sawhney, Pathak and Hubbell in Macromolecules, 26, 581 (1993) describe the preparation of block copolymers of polyglycolide or polylactide and PEG that are terminated with acrylate groups, as shown below:

$$CH_2=CH-CO-(O-CH_2-CO)_n-PEG-(O-CH_2-CO)_n-O-CO-CH=CH_2$$

In the above formula, the glycolide blocks are the $-O-CH_2-CO-$ units; addition of a methyl group to the methylene gives a lactide block; n can be multiples of 2. Vinyl polymerization of the acrylate groups produces an insoluble, crosslinked gel with a polyethylene backbone. The polylactide or polyglycolide segments of the polymer backbone, being ester groups, are susceptible to slow hydrolytic breakdown, with the result that the crosslinked gel undergoes slow degradation and dissolution.

Substantial non-PEG elements are introduced into the hydrogel. Non-PEG elements tend to introduce complexity into the hydrogel and degradation and dissolution of the matrix can result in undesirable or toxic components being released into the blood stream when the hydrogels are used in vivo for drug delivery.

It would be desirable to provide alternative PEG hydrogels that are suitable for drug delivery and that have unique properties that could enhance drug delivery systems.

Summary of the Invention

The invention provides chemically crosslinked PEG hydrogels for controlled release of conjugates of PEG and various molecules, including, for example, conjugates of PEG and enzymes, polypeptides, drugs, nucleosides, phospholipids, and other bioactive substances. The invention also provides methods for preparing the hydrogels.

The hydrogels of the invention are formed by reaction of active derivatives of poly(ethylene glycol) with amine groups on the bioactive substance or other molecule and with amine groups on other poly(ethylene glycol) molecules or related similar nonpeptidic polymers that typically do not contain hydrolytically unstable linkages. The poly(ethylene glycol) molecules that contain weak linkages in their backbones permit hydrolytic degradation of the crosslinks in the polymer

matrix and release of the bioactive substance with the other poly(ethylene glycol) or related nonpeptidic polymer attached. Degradation of the gel in vivo releases PEG/molecule conjugates into the blood stream and produces substantially nontoxic polymer fragments that typically are cleared from the body. Variation of the atoms near the hydrolytically unstable linkages can provide precise control of hydrolytic breakdown rate and release of the conjugate.

Examples of hydrolytically unstable linkages in the PEG polymer backbone include carboxylate ester, phosphate ester, acetals, imines, orthoesters, peptides, anhydrides, ketals, and oligonucleotides. These weak links are formed by reaction of two PEGs having different terminal groups as illustrated below:

$$\text{-PEG-Z} + \text{Y-PEG-} \longrightarrow \text{-PEG-W-PEG-}$$

In the above illustration, -W- represents the hydrolytically unstable weak link. Z- and Y- represent groups located at the terminus of the PEG molecule that are capable of reacting with each other to form weak links -W-. Examples of pairs of Z and Y groups that react to form hydrolytically unstable linkages W include pairs selected from the group consisting of alcohol, and carboxylic acid reacting to form carboxylate esters, amine and aldehyde reacting to form imines, hydrazide and aldehyde reacting to form hydrozones, alcohol and phosphate reacting to form phosphate ester, aldehyde and alcohol reacting to form acetals, alcohols and formate reacting to form orthoesters, peptides formed by the reaction of PEG amine with PEG-peptide terminated with carboxyl to form a new peptide linkage, peptides formed by the reaction of PEG carboxylic acid with PEG-peptide terminated wit amine to form a new peptide linkage, and oligonucleotides formed by reaction of PEG

phosphoramidite with an 5'-hydroxyl-terminated PEG oligonucleotide.

For example, the following pairs of Z and Y groups can be used to form some of the W groups described above:

$-PEG-CO_2H + HO-PEG- \longrightarrow$	$-PEG-CO_2-PEG-$	ester
$-PEG-OPO_3H_2 + HO-PEG \longrightarrow$	$-PEG-OPO_3(H)-PEG-$	phosphate ester
$-PEG-CHO + (HO-PEG)_2- \longrightarrow$	$-PEG-CH(O-PEG)_2-$	acetal
$-PEG-CHO + NH_2-PEG- \longrightarrow$	$-PEG-CH=N-PEG-$	imine

The PEG hydrogels gels are prepared by mixing three ingredients: (1) a PEG with hydrolytically unstable linkages W in the backbone and with reactive groups X at the ends of the chain, (2) a branched PEG or related nonpeptidic polymer with reactive groups Q at the ends of the chain, and (3) a bioactive molecule or other molecule containing reactive groups Q. Reactive groups X are selected from the group consisting of succinimidyl (NHS), as in $-O-(CH_2)_n-CO_2-NHS$ or $-O-CO_2-NHS$, and related activating groups, including sulfosuccinimidyl, benzotriazole, and p-nitophenyl. Reactive groups Q typically are amine, $-NH_2$.

A crosslinked network is produced that is held together by hydrolytically unstable groups W and groups T, which are hydrolytically stable. Hydrolysis of the unstable groups W releases the bioactive or other molecule with PEG or a related polymer attached, usually by a covalent linkage, which is hydrolyticlly stable.

The degree of branching of the polymers can be varied in the hydrogels of this invention to control the physical strength and compressibility of the gels. In general, the greater the degree of branching and the shorter the branches, the greater the strength of the gels, the smaller the pores, and the lower the water

content. Strength in this context is defined as resistance to compression or stretching.

The rate of release of molecules trapped within the hydrogel matrix is controlled by controlling the hydrolytic breakdown rate of the gel. The hydrolytic breakdown rate of the gel can be adjusted by controlling the degree of bonding of the PEGs that form the hydrogel matrix. A multiarm PEG having 10 branches or arms will break down and release drug molecules more slowly than a 3 arm PEG.

The following PEG has been made with two hydrolytically unstable ester linkages in its backbone:

$$NHS\text{-}O_2C\text{-}CH_2\text{-}O\text{-}PEG\text{-}O\text{-}CH_2\text{-}CO_2\text{-}PEG\text{-}O_2C\text{-}CH_2\text{-}O\text{-}PEG\text{-}O\text{-}CH_2\text{-}CO_2\text{-}NHS$$

The above PEG is activated at each terminus with an N-hydroxylsuccinimide moiety (NHS) in which the active succinimidyl ester moiety is $NHS\text{-}CO_2$- and is reactive with amino groups. A crosslinked network is produced that is held together by stable amide linkages and by hydrolytically unstable ester linkages when the above molecule is coupled with a multiarm PEG amine and with, for example, a protein that contains additional amino groups. The stable amide linkages are formed from reaction of the active NHS ester with amine.

The above example illustrates some of the advantageous features of the invention. First, the crosslinked network degrades or breaks down because of hydrolysis of the hydrolytically unstable ester linkages (W) in the PEG backbone. Second, when the gel breaks down, it releases PEG and protein conjugates, potentially useful for therapeutic application. Third, subtle variation of the ester linkage provides control over the hydrolytic breakdown rate.

In the above example the ester linkage has the following structure:

$$-PEG\text{-}O\text{-}CH_2\text{-}CO_2\text{-}PEG-$$

This ester group will hydrolyze with a half life of 4 days at pH 7 and 37°C. However, if an ester with the following structure is used, then the half life of hydrolytic degradation of the ester linkages is 43 days at pH 7 and 37°C.

$$-PEG-O-(CH_2)_n-CO_2-PEG- \qquad n = 2$$

Thus, by controlling the identity of the atoms adjacent to the ester linkage it is possible to vary the hydrolytic breakdown rate of the gel. Hence, it is possible to control the rate of release of PEG and protein conjugates bound within the matrix. In general, increasing the n value, which is the number of methylene groups in the above structure, decreases the hydrolysis rate.

Thus, the invention provides, among other things, degradable PEG hydrogels having hydrolytically unstable linkages in which the rate of hydrolysis of the unstable linkages can be controlled for release into the blood stream of conjugates of PEG or related nonpeptidic polymers and proteins or other molecules having some therapeutic effect.

The foregoing and other objects of the invention, and the manner in which the same are accomplished, will be more readily apparent upon consideration of the following detailed description of the invention taken in conjuction with the accompanying drawing, which illustrates an exemplary embodiment.

Brief Description of the Drawings

Figure 1 is a release profile from a PEG hydrogel prepared in accordance with the invention of a model protein (FITC-BSA) covalently linked to PEG.

Detailed Description

Hydrogels made from the crosslinked PEG polymeric structures of the invention can be used in drug delivery systems and for wound dressings. Wound dressings could be used internally to provide dressings that degrade within the body over time. The hydrogels

of the invention could be usefully applied in drug delivery systems to burns to apply polymer conjugated therapeutic agents to burns. Drug delivery systems can be prepared in which the rate of hydrolysis of the hydrogel is controlled to provide controlled release of drug components.

By "drug" is meant any substance intended for the diagnosis, cure, mitigation, treatment, or prevention of disease in humans and other animals, or to otherwise enhance physical or mental well being. The invention could be used for delivery of biologically active substances generally that have some activity or function in a living organism or in a substance taken from a living organism.

The terms "group," "functional group," "moiety," "active moiety," "reactive site," and "radical" are all somewhat synonymous in the chemical arts and are used in the art and herein to refer to distinct, definable portions or units of a molecule and to units that perform some function or activity and are reactive with other molecules or portions of molecules.

The term "linkage" is used to refer to groups that normally are formed as the result of a chemical reaction and typically are covalent linkages. Hydrolytically stable linkages means that the linkages are stable in water and do not react with water at useful pHs for an extended period of time, potentially indefinitely. Hydrolytically unstable linkages are those that react with water, typically causing degradation of a hydrogel and release of substances trapped within the matrix. The linkage is said to be subject to hydrolysis and to be hydrolyzable. The time it takes to degrade the crosslinked polymeric structure is referred to as the rate of hydrolysis and is usually measured in terms of its half life.

The skilled artisan should recognize that when reference is made to a Z moiety reacting with a Y

moiety, that additional reagents or steps may be employed according to commonly accepted chemical procedures and standards to achieve the desired linkage W as the case may be. There are many possible routes, too numerous to mention here, that could be taken and that should be readily apparent to the skilled artisan. For example, one of skill in the art can be expected to understand that when an alcohol and a carboxylic acid are reacted, the acid typically is converted to another form, the acid chloride, prior to reaction with alcohol. Several examples are demonstrated in the Examples below.

It should also be recognized that related branched nonpeptidic polymers that do not have hydrolytically unstable linkages can be used instead of the branched PEG polymer as an ingredient in the preparation of the hydrogels of the invention. These other branched polymers include poly(vinyl alcohol) ("PVA"); other poly(alkylene oxides) such as poly(propylene glycol) ("PPG") and the like; and poly(oxyethylated polyols) such as poly(oxyethylated glycerol), poly(oxyethylated sorbitol), and poly(oxyethylated glucose), and the like. The polymers can be homopolymers or random or block copolymers and terpolymers based on the monomers of the above polymers, straight chain or branched, or substituted or unsubstituted similar to mPEG and other capped, monofunctional PEGs having a single active site available for attachment to a linker.

Specific examples of suitable additional polymers include poly(oxazoline), poly(acryloylmorpholine) ("PAcM") as described in published Italian Patent Application MI-92-A-0002616 filed November 17, 1992, and poly(vinylpyrrolidone) ("PVP"). PVP and poly(oxazoline) are well known polymers in the art and their preparation and use in

the syntheses described with branched PEG should be readily apparent to the skilled artisan.

The following examples illustrate preparation of PEGs having hydrolytically unstable linkages in the polymer backbone and their use in preparing degradable hydrogels for the release of PEG and biomolecule conjugates. PEGs having hydrolytically unstable linkages and their preparation are also described in a copending patent application U.S.S.N. _____, entitled Degradable Poly(ethylene glycol) Hydrogels With Controlled Half-life and Precursors Therefor, which was filed on September 12, 1997 and claims priority from Provisional Application Serial No. 60/026,066, which was filed on September 13, 1996, the contents of which relating to the preparation of PEGs having hydrolytically unstable linkages in the polymer backbone are incorporated by reference in their entirety.

Examples

Example 1

Synthesis of PEG derivatives having hydrolytically unstable backbone linkages and terminal NHS active carbonates (NHS-OOCO-PEG-W-PEG-OCOO-NHS)

In a 100 ml round-bottom flask, benzyloxy-PEG carboxymethyl acid 3400 (3.4 g, 1mmol, Shearwater Polymers, Huntsville, AL) in toluene was azeotropically distilled for two hours and then cooled to room temperature. A solution of thionyl chloride (2M, 4 ml, 8 mmole, Aldrich) in methylene chloride was injected and the mixture was stirred under N_2 overnight. The solvent was condensed by rotary evaporation and the syrup was dried in vacuo for about four hours over P_2O_5 powder. To the residue was added anhydrous methylene chloride (5 ml) and azeotropically dried benzyloxy-PEG 3400 (2.55 g, 0.75 mmol) in toluene (20 ml). After the benzyloxy-PEG acyl chloride was dissolved, freshly distilled triethylamine (0.6 ml) was added. The mixture was stirred overnight, the triethylamine salt filtered off, and the product collected by precipitation with ethyl ether. It was further purified by dissolving in water and extracting with methylene chloride. The organic phase was dried over anhydrous sodium sulfate, condensed under vacuum, and precipitated into ethyl ether. The precipitate was dried in vacuo. HPLC (GPC) of the product showed that 100% of benzyloxy-PEG had been converted into the PEG ester and about 15 wt% benzyloxy-PEG acid remained.

The mixture was chromatographically purified on an ion-exchange column (DEAE sepharose fast flow, Pharmacia) to remove the benzyloxy-PEG acid. 100% pure α-benzyloxy-ϖ-benzyloxy PEG ester 6800 (2 g, 0.59 mmole end group) in 1,4-dioxane (20 ml) was hydrogenolyzed with H_2 (2 atm pressure) and Pd/C (1 g, 10% Pd) overnight. The catalyst was removed by filtration and the product precipitated into ethyl after most of the

solvent was removed on a rotary evaporator. α-hydroxy-ϖ-hydroxy PEG ester 6800 was collected by filtration and dried in vacuo. Yield: 1.5 gram (75%)

α-hydroxy-ϖ-hydroxy PEG ester 6800 (1.5 g, 0.44 mmole end group) was azeotropically dried with 100 ml of acetronitrile and cooled to room temperature. To this solution was added disuccimidyl carbonate (DSC) (0.88 mmole, Fluka) and pyridine (0.1 ml), and the solution was stirred at room temperature overnight. The solvent was removed under vacuum and the syrup was dried in vacuo. The product was dissolved in 35 ml of dry methylene chloride, the insoluble solid was removed by filtration, and the filtrate washed with pH 4.5 sodium chloride saturated acetate buffer. The organic phase was dried over anhydrous sodium sulfate, condensed under vacuum, and precipitated into ethyl ether. The precipitate was dried over P_2O_5 in vacuo. Yield: 1.4 g (93%). NMR (DMSO-d_6): (1) product from benzyloxy-PEG propionic acid: δ 3.5 (br m, PEG), 2.55 (t, -$OCH_2\underline{C}H_2COOPEG$-), 4.13 (t, -PEG-$COO\underline{C}H_2CH_2O$-), 4.45 (t, -$PEGOCH_2\underline{C}H_2OCO$-NHS), 2.80 [s, NHS, 4H]; (2) product from benzyloxy-PEG carboxymethyl acid: δ 3.5 (br m, PEG), 4.14 (s, -$OC\underline{H}_2COOPEG$-), 4.18 (t, -$OCH_2COOC\underline{H}_2CH_2$-), 4.45 (t, -$PEGO-CH_2C\underline{H}_2OCONHS$), 2.81 [s, NHS, 4H].

Example 2

Synthesis of PEG derivatives having hydrolytically unstable backbone linkages and terminal NHS active esters

(NHS-OOC-$(CH_2)_n$-O-PEG-O-$(CH_2)_n$-CO_2-PEG-O_2C-$(CH_2)_n$-O-PEG-O-$(CH_2)_n$-COONHS)

In a 100 ml round-bottom flask, difunctional PEG 2000 (2 g, 1mmol, Shearwater Polymers) and difunctional PEG acid 2000 (4 g, 2 mmole, Shearwater Polymers) were azeotropically distilled with 70 ml of toluene under N_2. After two hours, the solution was cooled to room temperature and stannous 2-ethylhexanoate (200 mg, Sigma Chemical) was added. The solution was then refluxed under N_2 for 24 hours. The solvent was then condensed under vacuum and the syrup

precipitated into 100 ml of ether. The product was collected by filtration, dried under vacuum, and dissolved in a sodium acetate buffer solution at pH 5.0. The slightly milky solution was centrifuged and the upper clear solution was extracted three times with methylene chloride. The organic phase was dried over anhydrous sodium sulfate, filtered, condensed under vacuum, and precipitated into ether. The product was collected by filtration and dried under vacuum. HPLC: 70% product, 15% di-acid reactant and 15% monoacid. The mixture was further purified by ion exchange chromatography and gel permeation chromatography. Yield 3 g (50%). ^{1}H NMR (DMSO-D_6): (1) product from PEG carboxymethyl acid: δ 3.5 (br m, PEG), 4.15 (s, -OC$\underline{H}_2$COOCH_2-), 4.18 (t, -OCH_2COOC$\underline{H}_2$CH_2-), 3.98 (s, -PEG-OC$_{H2}$COOH); (2) product from PEG propionic acid: δ 3.5 (br m, PEG), 2.55 (t, -PEGOCH_2C$\underline{H}_2$COOCH_2-), 4.13 (t, -OCH_2CH_2COOC$\underline{H}_2$CH_2-), 2.43 (t, -PEGOCH_2C$\underline{H}_2$COOH).

In a round-bottom flask, the difunctional acid having weak linkages (obtained from previous step) 3 g. approx. 1 mmole end group) and N-hydroxysuccinimide (NHS) (126 mg, 1.05 mmole) were dissolved in 50 ml of dry methylene chloride. To this solution was added dicyclohexylcarbodiimide (240 mg, 1.15 mmole) in 5 ml dry methylene chloride. The mixture was stirred under N_2 overnight. The solvent was condensed and the syrup was redissolved in 15 ml of anhydrous toluene. The insoluble salt was removed by filtration and the filtrate was precipitated into 200 ml of dry ethyl ether. The precipitate was collected by filtration and dried in vacuo. Yield 2.7 g (90%). ^{1}H NMR (DMSO-d_6): δ 3.5 (br m, PEG), 2.8 (s, NHS, 4H), 4.6 (s, -PEG-O-C$\underline{H}_2$-COONHS) or 2.85 (t, -PEG-O-CH_2C$\underline{H}_2$-COONHS).

Example 3

Hydrolysis kinetics of the ester linkages in the middle of the PEG derivatives

To precisely measure the hydrolysis kinetics of the ester linkages, water-soluble, non-crosslinked mPEG-O-$(CH_2)_n$-COO-PEGm was synthesized as in Example 2. Hydrolysis was carried out in buffer solutions (0.1 M) at different pHs and temperatures, and followed by HPLC-GPC (Ultrahydrogel® 250, Waters). The half-lives of the ester bonds are listed in Table 1.

Table 1

Hydrolysis half lives (days, ±10%) of the ester of mPEG-O-$(CH_2)_n$-COO-PEGm in 0.1 M phosphate buffer.

	PA ester linkage			CM ester linkage		
pKa of the acid	4.45±0.1			3.67±0.05		
pH	5.5	7.0	8.1	5.5	7.0	8.1
Room Temp. (22-23°C)	>500	250	37	>150	30	5
37°C		43			4	
50°C		15			1.5	

Example 4

Preparation of a hydrolytically unstable PEG hydrogel from branched PEG amine, model protein (FITC-BSA) and PEG derivatives having hydrolytically-unstable backbone linkages and terminal NHS active carbonates (NHS-OOCO-PEG-W-PEG-OCOONHS)

In a test tube, 100 mg (14.7 μmole) of difunctional PEG active carbonate 6800 (NHS-OOCO-PEG-W-PEG-OCOONHS, prepared in Example 1) was dissolved in 0.75 ml of buffer (0.1M phosphate, pH 7). To the solution were added 0.15 ml of 8-arm-PEG-amine 10000 (250 mg/ml) and 0.1 ml of FITC-BSA (10 mg/ml). After rapid shaking, it was allowed to sit and a gel formed in a few minutes. A suitable buffer pH range was found to be 5.5 to 8.

Example 5

Preparation of a hydrolytically unstable PEG hydrogel from branched PEG amine, model protein, and PEG derivatives having hydrolytically unstable backbone linkages and terminal NHS active esters (NHS-OOC-$(CH_2)_n$-O-PEG-O-$(CH_2)_n$-CO_2-PEG-O_2C-$(CH_2)_n$-O-PEG-O-$(CH_2)_n$-COONHS)

100 mg (approx. 16.6 μmole) difunctional PEG active ester (NHS-OOC-$(CH_2)_n$-O-PEG-O-$(CH_2)_n$-CO_2-PEG-O_2C-$(CH_2)_n$-O-PEG-O-$(CH_2)_n$-COONHS, prepared in Example 2) was dissolved in 0.75 ml of buffer (0.1M phosphate, pH 7). To the solution were added 0.166 ml of 8-arm-PEG-amine 10000 (250 mg/ml) and 0.1 ml of FITC-BSA (10 mg/ml). After rapid shaking, it was allowed to sit and a gel formed in a few minutes. A suitable buffer pH range was found to be 5.5 to 8.

Example 6

Studies of release of model proteins from hydrolytically degradable hydrogels

All protein-loaded hydrogel disks were weighed and their diameters measured before release studies. Then each gel disk was immersed, at time t=0, in phosphate buffer (0.1 M, pH 7.0). The amount of the buffer was more than 50 times that of the wet gel weight. The solution was maintained at 37°C, and gently shaken. At a predetermined time, a small amount of buffer solution was removed for protein concentration determination and then put back after measurement. The protein concentration was determined by UV measurement at 495 nm. Figure 1 shows some release profiles of PEG-FITC-BSA from the hydrogels in units plotted against time in days of the fraction of moles at time t divided by the moles at infinity, which is defined as the completion of degradation of the hydrogel.

The invention has been described in particular exemplified embodiments. However, the foregoing description is not intended to limit the invention to the exemplified embodiments, and the

skilled artisan should recognize that variations can be made within the scope of the invention as described in the foregoing specification. The invention includes all alternatives, modifications, and equivalents that may be included within the true spirit and scope of the invention as defined by the appended claims.

THAT WHICH IS CLAIMED IS:

1. A crosslinked polymeric structure that degrades in aqueous solution and releases into the solution conjugates of a bioactive agent and a substantially nonpeptidic polymer.

2. The structure of Claim 1 wherein the conjugate is degradable in aqueous solution.

3. A crosslinked polymeric structure containing segments of the formula poly-T-poly-W-PEG-W-poly-T-D, wherein PEG is a branched or linear polyethylene glycol of molecular weight from 300 to 200,000 daltons; poly is a polymer selected from the group consisting of poly(alkylene oxides), poly(oxyethylated polyols), poly(olefininc alcohols), and poly(acrylomorpholine); W is a hydrolytically unstable linkage selected from the group consisting of carboxylate ester, phosphate ester, orthoester, anhydride, imine, acetal, ketal, oligonucleotide, peptide; T is a hydrolytically stable group selected from the group consisting of amide, urethane, amine, ether, thioether, and urea; and D is a biologically active molecule.

4. The polymeric structure of Claim 2 wherein the biologically active molecule D is selected from the group consisting of enzymes, polypeptides, drugs, nucleosides, and phospholipids.

5. The polymeric structure of Claim 2 wherein poly is selected from the group consisting of poly(ethylene glycol), poly(oxyethylated glycerol), poly(oxyethylated sorbitol), poly(oxyethylated glucose), poly(vinyl alcohol), and poly(propylene glycol).

6. A method of preparing a crosslinked polymeric structure that hydrolyzes to release conjugates of a biologically active molecule with a substantially non-peptidic polymers by reacting (1) PEGs with hydrolytically weak linkages in their backbones, (2) branched, substantially non-peptidic polymeric amines, and (3) biologically active molecules to form the structure.

7. The method of Claim 5 wherein the reaction can be represented by the following:

$$X\text{-PEG-W-PEG-X} + R(CH_2\text{-O-poly-}NH_2)_p + D\text{-}NH_2) \longrightarrow \text{product}$$

wherein

X is selected from the group consisting of succinimidyl ester, sulfosuccinimidyl, benzotriazole, and p-nitrophenyl;

R is a central branching group leading to branched polymers poly that is selected from the group consisting of glycerol, glycerol oligomers, pentaerythritol, sorbitol, trimethyolpropane, and di(trimethylolpropane);

p = 3 to 10 and represents to the degree of branching of the branched polymer poly;

poly is a polymer selected from the group consisting of poly(alkylene oxides), poly(oxyethylated polyols), poly(olefininc alcohols), and poly(acrylomorpholine);

W is a hydrolytically unstable linkage selected from the group consisting of carboxylate ester, phosphate ester, orthoester, anhydride, imine, acetal, ketal, oligonucleotide, and peptide; and

D is a biologically active molecule.

8. The method of Claim 7 wherein X is $-O-(CH_2)_n-CO_2-NHS$ or $-O-CO_2NHS$, and wherein n = 1-10.

9. The method of Claim 7 wherein W is an ester linkage $-O-(CHR')_r-CO_2-$, wherein r = 1 through 10, and R'is hydrogen or alkyl.

10. The method of Claim 6 wherein the branched, substantially non-peptidic polymeric amines do not have weak linkages in their backbones.

11. The method of Claim 7 wherein poly is selected from the group consisting of poly(alkylene oxides), poly(vinyl pyrrolidone), poly(vinyl alcohol), polyoxazoline, and poly(acryloylmorpholine).

12. A system for the delivery in vivo or to a substance taken from living tissue of conjugates of substantially non-peptidic polymers with biologically active molecules comprising the polymeric structure of Claim 6.

13. A process for applying therapeutic agents to wounds and scars, said process comprising applying to the wound or scar a crosslinked polymeric structure as recited in Claim 1, wherein said biologically active molecule is said therapeutic agent.

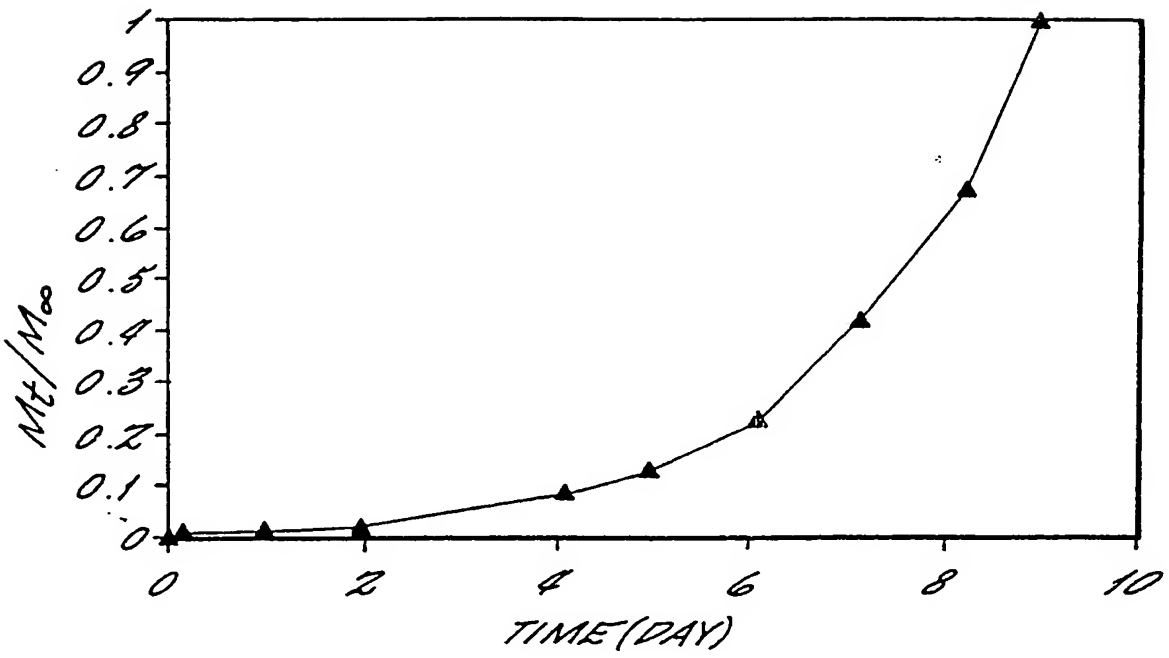

Fig. 1.

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 98/00918

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 21469 A (SHEARWATER POLYMERS INC ;HARRIS J MILTON (US); VERONESE FRANCESCO) 18 July 1996 see abstract see drawings page 32-41 see page 49, line 8 - page 50, line 4 see page 65, line 20-35; claims 2,8-12,18,19,36,37; figure 2B ---	1-13
X	MARTINEZ A ET AL: "BRANCHED POLY(ETHYLENE GLYCOL) LINKERS" MACROMOLECULAR CHEMISTRY AND PHYSICS, vol. 198, no. 8, August 1997, pages 2489-2498, XP000699067 see abstract; figure 1 see page 2490, paragraph 1 see page 2498, paragraph 1 --- -/--	1-7,9-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1998	02/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gonzalez Ramon, N

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	JEONG B ET AL: "BIODEGRADABLE BLOCK COPOLYMERS AS INJECTABLE DRUG-DELIVERY SYSTEMS" NATURE, vol. 388, no. 6645, 28 August 1997, pages 860-862, XP002068338 see abstract see page 861, paragraph 4 - page 862, paragraph 2 ---	1-5,13
X	SAWHNEY A S ET AL: "BIOERODIBLE HYDROGELS BASED ON PHOTOPOLYMERIZED POLY(ETHYLENE GLYCOL)-CO-POLY(A-HYDROXY ACID) DIACRYLATE MACROMERS" MACROMOLECULES, vol. 26, no. 4, 15 February 1993, pages 581-587, XP000360803 see conclusions see abstract; figures 1,7 see page 586, column 2 ---	1-5,13
X	WO 92 00748 A (ENZON INC) 23 January 1992 see abstract see page 52-57; claims 57,58; examples 3,5,13-16,19 ---	1-7, 10-13
X	WO 93 24476 A (CLOVER CONS LTD ;SOON SHIONG PATRICK (US); DESAI NEIL P (US); SAND) 9 December 1993 see abstract; claims 2,3,6-812-14; examples 3,7 see page 12, line 20 - page 13 ---	1-13
Y	ZALIPSKY S: "FUNCTIONALIZED POLY(ETHYLENE GLYCOL) FOR PREPARATION OF BIOLOGICALLY RELEVANT CONJUGATES" BIOCONJUGATE CHEMISTRY, vol. 6, no. 2, 1995, pages 150-165, XP002068523 see abstract see page 153, column 2, paragraph 3 - page 155, column 1; figures 4,5 see page 161, paragraph 2 ---	1-13
Y	PITT C G ET AL: "Manipulation of the rate of hydrolysis of polymer-drug conjugates: The secondary structure of the polymer" JOURNAL OF CONTROLLED RELEASE, vol. 39, no. 2, May 1996, page 221-229 XP004037328 see abstract; figures 4,5 --- -/--	1-13

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 98/00918

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y,P	EP 0 841 360 A (ETHICON INC) 13 May 1998 see page 9, line 42 - page 10, line 6; claim 12; examples 2,4	1-13
A	EP 0 473 268 A (ICI PLC) 4 March 1992 see abstract; claims 1,7,8; examples 19-22	1-13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/US 98/00918

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 12-13 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 12-13
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Rule 39.1 (iv) PCT - Method for treatment of the human or animal body by therapy

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 98/00918

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9621469 A	18-07-1996	AU 4755596 A	31-07-1996
WO 9200748 A	23-01-1992	US 5455027 A	03-10-1995
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WO 9324476 A	09-12-1993	AU 4406793 A	30-12-1993
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